PB-229 194

EFFECTIVENESS OF HALOGENS OR HALOGEN-CONTAINING COMPOUNDS IN DETOXIFYING 'CLOSTRIDIUM BOTULINUM' TOXINS

A. Richard Brazis, et al

Robert A. Taft Sanitary Engineering Center

Prepared for:

Bureau of Yards and Docks

March 1958

DISTRIBUTED BY:

NTIS

National Technical Information Service
U. S. DEPARTMENT OF COMMERCE
5285 Port Royal Road, Springfield Va. 22151

PB 229 194

EFFECTIVENESS OF HALOGENS OR HALOGEN-CONTAINING COMPOUNDS IN DETOXIFYING *CLOSTRIDIUM BOTULINUM* TOXINS

A report to the Department of the Navy,
Bureau of Yards and Docks, prepared under
Project NY 300 010 Subproject 6

A. Richard Brazis, Alton R. Bryant,
James E. Leslie, Richard L. Woodward,
and Paul W. Kabler
Water Supply and Water Pollution Program

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service
Bureau of State Services
Division of Sanitary Engineering Services

Robert A. Taft Sanitary Engineering Center
Cincinnati 26, Ohio

March, 1958

Reproduced by
NATIONAL TECHNICAL
INFORMATION SERVICE
U S Department of Commerce
Springfield VA 22151

CONTENTS

	Page
ABSTRACT	iii
SUMMARY	iv
INTRODUCTION	1
REVIEW OF LITERATURE	1
MATERIALS AND METHODOLOGY	5
Toxin Assay	6
Routine Determinations	8
Test Procedure	11
RESULTS	12
Toxin Stability	12
Free Available Chlorine	16
Combined Chlorine	16
Globaline	24
Chlorine Dioxide	24
DISCUSSION	24
Effect of Temperature	24
Effect of pH	29
Effect of Free Available Chlorine Concentration	31
Effect of Combined Chlorine	35
Effect of Iodine Concentration	37
Effect of Chlorine Dioxide Concentration	38
Variations in Toxin Sensitivity	39
CONCLUSIONS	40
REFERENCES	42

ABSTRACT

The effectiveness of halogens or halogen-containing compounds in destroying purified and partially purified Clostridium botulinum toxins, under varying conditions of pH, temperature, and length of exposure, has been evaluated. The detoxifying substances studied were free chlorine, chloramine, Globaline, and chlorine dioxide.

Free available chlorine in concentrations sufficient to provide residuals of 1 mg/l will destroy at least 99.9 percent of all 5 types of botulinum toxin in five minutes or less. Chlorine dioxide is somewhat more effective than free available chlorine in destroying the toxin most resistant to chlorine. Monochloramine, dichloramine, and chloramine-T destroy the toxin only on prolonged contact with relatively high concentrations. Globaline tablets will destroy the type A toxin within 60 seconds when used as recommended for disinfection of water in canteens.

The type E toxin is the most resistant of the five types to destruction by chlorine.

SUMMARY

Effectiveness of several halogens in destroying pure and partially purified toxins of Clostridium botulinum was evaluated at temperatures of 5° and 25°C., and at pH levels of 7.0, 8.0, 9.0, and 10.0 as follows:

1. All toxins evaluated were stable in the test waters used, at both test temperatures, in the absence of halogens under the conditions of the investigation.
2. Toxin concentrations were derived from log-log graphs denoting LD_{50}s present versus minutes required for 50 percent kill of inoculated mice.
3. Free available chlorine, in initial concentrations of 0.5 mg/l, produced 99.9 to 99.99 percent inactivation of the partially purified type A toxin at all pH levels and temperatures indicated, within 15 to 30 seconds.
4. The types B and C toxins (partially purified) required residuals of 0.46 and 0.20 mg/l free available chlorine, respectively, for 99.9 percent inactivation in 15 to 30 seconds, at pH 7.0 and 25° C. The high chlorine demand of these two toxins necessitated the addition of high free available chlorine concentration initially.
5. The purified type D toxin was 99.9 to 99.99 percent inactivated at pH 7.0 with storage at 5° and 25° C., within 2 minutes, when 0.5 mg/l free available chlorine was added.
6. The type E undialysed crude toxin, although unstable at pH levels above 8.0, required a residual of 0.87 mg/l free available chlorine due to its high chlorine demand, to

produce 99.9 to 99.99 percent inactivation in 1 to 2 minutes, at pH 7.0 and 25° C.

7. Monochloramine required 4 hours for 99.96 percent inactivation of the partially purified type A toxin, in the presence of 11.6 mg/l at pH 7.0 and 25° C.

8. Monochloramine-T produced a 99.99 percent inactivation of the partially purified type A toxin at pH 7.0 and 25° C., within 7 minutes, when 11.7 mg/l were used.

9. Globaline tablets containing 6.9 to 7.7 mg/l iodine per tablet when dissolved in one quart of water at 25° C., produced 99.9 to 99.99 percent inactivation of the partially purified type A toxin within 60 seconds at pH 6.3.

10. A chlorine dioxide concentration residual of 0.18 mg/l produced 99.9 to 99.99 percent inactivation of the crude undialysed type E toxin in 15 to 30 seconds at pH 7.0 and 25° C. The purified type E toxin was 99.9 to 99.99 percent inactivated within 10 to 15 seconds, in the presence of 0.27 mg/l chlorine dioxide, under the same conditions.

EFFECTIVENESS OF HALOGENS OR HALOGEN-CONTAINING COMPOUNDS IN DETOXIFYING *CLOSTRIDIUM BOTULINUM* TOXINS

INTRODUCTION

Botulinum toxin is not normally present in water and has not posed any public health hazard by this vehicle. Five types of botulinum toxin are known. Although they have been extensively studied because of their importance as food poisons, little information is available about their behavior in water and the effectiveness of water treatment in destroying the toxin.

Because of their importance as possible agents for intentional contamination of water, this study was undertaken to find suitable methods for protecting against such contamination. Information in the literature indicated that oxidizing agents would destroy the toxin. However, the available data were insufficient to provide a suitable basis for design of protective measures.

REVIEW OF LITERATURE

Lamanna, McElroy and Eklund[1] produced and purified *Clostridium botulinum* type A toxin in 1946. They used the Hall strain of *Cl. botulinum* type A. It has been shown that this strain produces the most potent type A toxin. The toxin culture was grown in a medium adjusted to pH 7.2 before sterilization and incubated at 34° C. for 72 to 80 hours. Acid treatment of the medium precipitated the toxin and the supernatant was siphoned off followed by purification of the toxin. Following crystallization, the toxin was stored in acetate buffer at pH 5.5 to 7.1.

Shaking with chloroform was later added as a step in the purification process by Lamanna, Eklund and McElroy[2]. Abrams, Kegeles, and Hottle[3] also prepared Cl. botulinum type A toxin in a manner similar to Lamanna et al[1], but used charcoal-treated pepticase in order to produce more uniform toxin production. More recently, Duff, Wright, Klerer, and Moore[4] have developed easier methods for producing and purifying the A toxin. The Hall strain was grown in a medium consisting of an enzymatic hydrolysate of casein, autolysed yeast extract, and glucose. After 4 days' growth, titers approximating 1.5×10^6 mouse LD_{50}/ml were obtained. Following precipitation and purification of the toxin in 5 stages, the toxin contained approximately 236×10^6 mouse LD_{50}/mg of nitrogen.

The number of minimum lethal doses per ml was determined by Abrams et al[3] by injecting 20-gram mice intraperitoneally with 1-ml volumes of the toxin diluted in 0.2 percent gelatin, buffered at pH 6.8. Their definition of 1 minimum lethal dose was the smallest amount of toxin which caused death within 4 days in all mice injected. In the work of Duff et al[4], the toxin was diluted in a buffer containing 0.2 percent gelatin and 0.4 percent dibasic sodium phosphate, adjusted to pH 6.8 with hydrochloric acid prior to injection into 18 to 20 gram mice. Intraperitoneal injections of 0.5-ml aliquots of the dilution of the toxin were used and the mice were observed over a 4-day period. Although 0.5-ml amounts of the toxin dilutions were used, LD_{50} results were expressed in terms per ml.

Assays of the A toxin were performed by many groups using different procedures of diluting the toxin prior to mouse injections, with widely varying results as to the toxicity of given preparations of the toxin.

Boor, Tresselt and Schantz[5] showed as much as a 50-fold variation in the toxicity determinations resulting from the use of various diluents such as sheep serum, sheep albumin serum, human albumin serum, gastric mucin, and gelatin. They concluded that a standardized diluent such as an autoclaved solution of 0.2 percent gelatin in 1.0 percent phosphate buffer must be chosen to obtain comparable results. The authors suggested that mucin, gelatin, serum proteins, etc., rendered the test animals more susceptible to the action of the toxin. Lamanna, Jensen, and Bross[6] found that the type A toxin was not affected by a difference of weights of mice of 6 to 12 grams or 20 to 25 grams. The other toxins examined showed small differences. Attempts to study the toxicity of the botulinum toxin by other than mouse assays has been pursued by Lowenthal and Lamanna[7]. Their work concerned the factors affecting the botulinal hemagglutination reaction and the relationship between hemagglutinating activity and toxicity of toxin preparations. The results of these experiments indicate a lack of identity of hemagglutinating activity with the toxicity of the Cl. botulinum type A culture supernates.

The resistance of botulinum toxins to dilution in water was studied by Brygoo[9]. The type A toxin in dilution appeared to be the most fragile. No mention was made of the purity of the toxin preparation based on the amount of nitrogen present per ml of toxin liquid. He concluded that botulinum toxins diluted in water, exposed to light and ordinary temperatures (no mention of hydrogen-ion concentration) can retain an important fraction of their lethal power over a relatively long time. This resistance was particularly marked for the type C toxin. The contamination of ponds used to water cattle could consequently play a role in the etiology of animal botulism.

Brygoo[10] reported that the 5 botulinum toxins are destroyed in 15 minutes by permanganate, hypochlorite, and iodide in the concentrations ordinarily used for the sterilization of water. From the results indicated in his report, all reported chlorine determinations were in terms of total chlorine. The effective chlorine concentration was considered as that which is capable of destroying in 15 minutes at laboratory temperature at least 80 percent of the toxin, which is defined by the survival of all inoculated mice. Mice were inoculated in lots of 3. The toxins were used at a toxicity level of 10 MLD/ml in all experiments. Based on conversion of grams per liter to milligrams per liter, their results indicated that the 5 toxins were "destroyed" by the corresponding amounts of chlorine: A-10 mg/l Cl_2; B-500 mg/l Cl_2; C-50 mg/l Cl_2; D-50 mg/l Cl_2; and E-500 mg/l Cl_2. No mention was made of the pH reactions or temperatures used in this investigation.

In a more recent paper, D'Arca and D'Arca[11] have described the effects of free "active" chlorine upon the type A botulinum toxin. From the results indicated, it appeared that the authors used combined chlorine rather than free available chlorine in destroying the type A toxin. Using toxin titers of 1, 5, and 10 MLDs, their results indicated that more chlorine was required to destroy the toxin than had been previously reported by Brygoo[10]. At the 10 MLD level, 12.5 to 15 mg/l free active chlorine were required when the type A toxin was held at ambient temperature for 60 minutes.

Although chlorine dioxide has never been tested against toxins, some mention should be made of its disinfecting efficacy. Ridenour, Ingols, and Armbruster[14, 15, 16, 17] have investigated its action against bacterial

spores, vegetative cells of Escherichia coli, and mouse-adapted poliomyelitis virus. Their investigations, although questionable in relation to chlorine dioxide measurement, appeared to indicate that chlorine dioxide was much more efficient than free available chlorine.

MATERIALS AND METHODOLOGY

Partially purified and pure forms of Cl. botulinum types A and D toxins used in this study were obtained from Dr. E. J. Schantz and Mr. James Duff of Fort Detrick, Frederick, Maryland. The partially purified form of the A toxin was received at this station with the following identification:

pH	3.9
Total Mouse Units (LD_{50})	1.6×10^{10}
Mouse Units per ml	6.8×10^{8}
Mouse Units per mg solids	1.1×10^{7}

The toxin was kept in its original container at 4°C until diluted in standard phosphate buffered distilled water prior to use. The toxin was diluted at the rate of 1 ml of original material per 99 ml of buffered distilled water. Thus a toxin stock mixture of approximately 5×10^{6} mouse intraperitoneal LD_{50} per ml was retained for further dilution into test flasks.

The pure form of the type A toxin contained 150×10^{6} mouse I.P. LD_{50} per ml; 0.65 mg N/ml, and 230×10^{6} LD_{50}/mg N. The toxin was dissolved in 0.05 molar acetate buffer, pH 3.8. The pure form of the toxin was also diluted to approximately 5.0×10^{6} mouse I.P. LD_{50} per ml and stored at 4°C. until ready for use. In the case of the diluted pure form

of the A toxin, the mixture was used approximately one week after its dilution into buffered distilled water. Mouse assays were continually made to insure knowledge of the degree of toxicity present in the stock toxin mixture.

Type D toxin was received at a strength of 150×10^6 mouse I.P. LD_{50}/ml. The pure form of the toxin was stored in glycerol at the time of arrival. This toxin was likewise diluted to approximately the same titer as the A toxin and stored at 4°C. Type B, C, and E toxins were received and processed similarly. The pH of all stored toxin mixtures ranged from 6.8 to 7.0.

TOXIN ASSAY

The partially purified Cl. botulinum type A toxin was assayed by injection into mice. In early assays, mice were inoculated intraperitoneally with 0.5 ml and in later assays with 0.6 ml. The toxin was assayed using the following diluents: (1) 0.4 percent dibasic sodium phosphate and distilled water; (2) .02 percent gelatin, 0.4 percent dibasic sodium phosphate, and distilled water; and (3) 0.2 percent gelatin, 0.4 percent dibasic sodium phosphate, 0.2 percent sodium thiosulfate, and distilled water. All diluents were adjusted to pH 6.8 prior to sterilization.

The use of the 3 different types of diluents confirmed the results of Boor, Tresselt and Schantz[5]. Table I shows the survival time in minutes of mice after injection of predetermined LD_{50} levels of type A toxin. When dibasic sodium phosphate and distilled water were used as the diluent, the toxin produced earlier death times with high

TABLE I. EFFECT OF DILUENTS UPON SURVIVAL TIME OF MICE INOCULATED WITH VARIOUS DOSES OF CL. BOTULINUM TYPE A TOXIN

Dilution	LD_{50}/inoculation	Survival time in minutes *		
		Diluent A	Diluent B	Diluent C
1:50	80,000	-**	57	52
1:100	40,000	47	73	59
1:200	20,000	51	70	77
1:400	10,000	62	84	92
1:800	5,000	79	94	91
1:1600	2,500	101	118	115
1:3200	1,200	209	131	121
1:6400	600	279	154	L.A.
1:13,000	300	512	160	137
1:26,000	150	666	175	183
1:53,000	75	1134	246	248
1:120,000	32	---	280	256
1:250,000	16	---	390	295
1:500,000	8	---	489	472
1:1,000,000	4	---	990	508
1:2,000,000	2	---	2876	1040
1:4,000,000	1	---	---b	1923
1:8,000,000	1	---	---	---b

*A Disodium phosphate and distilled water, adjusted to pH 6.8.
B Disodium phosphate, gelatin, and distilled water, adjusted to pH 6.8.
C Disodium phosphate, gelatin, sodium thiosulfate, and distilled water, adjusted to pH 6.8.
** Death time was not measured.
L.A. Laboratory accident.
--- Greater than the four-day observation period.

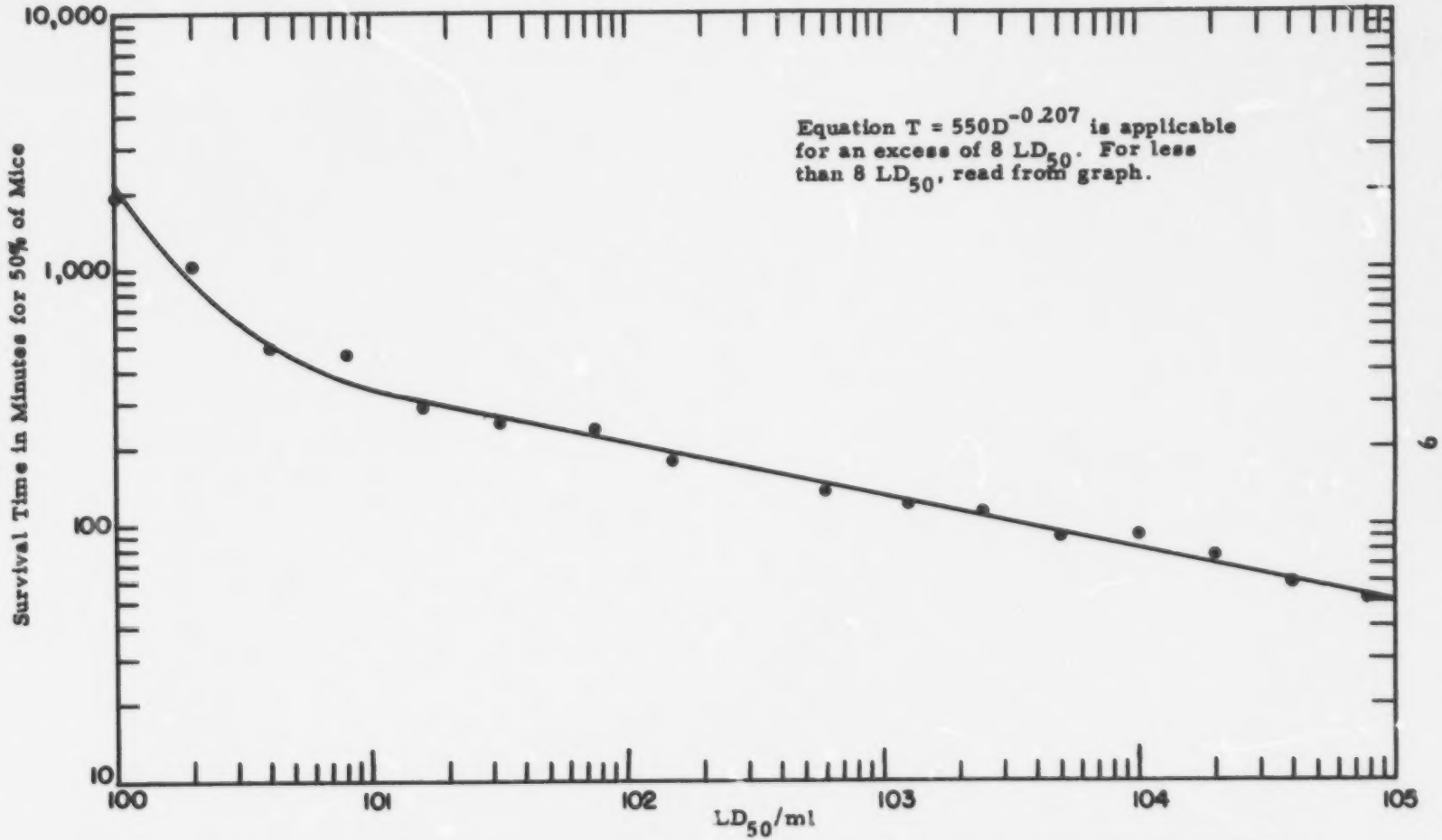
toxin doses than were seen for the other diluents tested. However, at doses below 2500 LD_{50}, the survival time was considerably longer than with the other diluents. At doses below 75 LD_{50} all mice survived when the diluent without gelatin was used, while as little as 1 LD_{50} was lethal when gelatin was added to the diluent.

The survival times were similar when diluents containing gelatin with and without sodium thiosulfate were used. Only at the level of 4 LD_{50}, or less, per inoculation, were significant differences noted. Since sodium thiosulfate must be present to neutralize the halogen being investigated, it was decided to incorporate the gelatin and dibasic sodium phosphate with the neutralizer, thus eliminating one step in preparing the toxin for inoculation. Diluent #3 was used in all subsequent assays.

In order to reduce the number of animals required for the study, most of the toxin assays on treated water were made by observing the survival time of mice after injection with the material of unknown toxicity. It was found that the relationship between survival time and toxin dose is sufficiently reproducible to serve as a method of assay. A calibration curve was prepared for partially purified type A toxin diluted in the thiosulfate diluent mentioned above, using 18 dilutions ranging from 80,000 LD_{50} per ml to less than 1 LD_{50} per ml. Figure 1 is a graph of the results of this dose-survival time study. Similar curves were prepared for estimation of toxin concentration of the types B, C, D, and E toxins.

ROUTINE DETERMINATIONS

Methods used in all hydrogen-ion concentration determinations, maintenance of storage temperatures, preparation of stock chlorine mixtures,



GRAPHIC PRESENTATION OF RELATIONSHIP BETWEEN LD_{50} OF CLOSTRIDIUM BOTULINUM TYPE A TOXIN AND THE SURVIVING TIME OF 50% OF INOCULATED MICE

FIGURE 1

synthetic test waters and buffers have been described in a previous report[13]. The determination of combined chlorine concentration was made according to the standard orthotolidine test[12].

Measurements of free available chlorine concentration were made by a modification of the orthotolidine-arsenite (OTA) method[12]. It was found that in the presence of high concentrations of ammonia or other nitrogenous materials, the normal procedure gave free chlorine determinations which were higher than were actually present. This result was due to some color development at 20° C. by combined chlorine during the 5-second period before addition of the arsenite. This difficulty was overcome by reducing the temperature of the water to 5° C. prior to addition of orthotolidine and arsenite.

Iodine concentration was determined by the starch-iodometric method[12].

Chlorine dioxide concentration was measured according to the procedure developed by Post and Moore[18]. The sample is allowed to react with malonic acid which removes free available and combined chlorine from the sample when present. Acetate buffer, ferric chloride and 1-amino, 8-naphthol, 3-6 disulfonic acid are the reagents used for the development of color.

All glassware was cleaned in a sulfuric acid-dichromate mixture, rinsed with distilled water, immersed in haemo-sol solution, followed by several rinsings with distilled water and chlorine demand-free distilled water, and sterilized in a hot air oven.

TEST PROCEDURE

Preceding each determination, 1- and 2-liter capacity Erlenmeyer flasks were prepared, containing twice the desired concentration of toxin and halogen. At the start of each experiment (0 seconds) 500 ml of toxin-water was added to 500 ml of halogen-water and was mechanically stirred.

During the tests for efficacy of free available chlorine, chlorine dioxide, and Globaline, samples were taken at intervals of 15 and 30 seconds, and at 1, 2, 4, 8, 16, 30, and 60 minutes for toxin assay. In those investigations involving chloramines, samples were taken at intervals of 3, 7.5, 15, 30 minutes, and 1, 2, and 4 hours. Preliminary studies, in which the rapid destruction rate of toxin by chlorine was not known, required intervals of sampling similar to those used during the chloramine evaluation tests.

At each sampling interval, 5 ml of the test water containing the toxin and the halogen were placed in neutralizer tubes containing the specific diluent. Male white Swiss mice, 18 to 20 gram weight, were inoculated in lots of 6 each with 0.6 ml of the neutralized sample. One-ml tuberculin syringes were used for inoculating the test and control mice. At least 3 lots of mice were inoculated with toxin test water not containing halogens, during each observation.

Times of inoculation and death of each lot of mice were recorded and used in the determination of toxin concentration at each interval of sampling. All mice were observed over a 4-day period. All surviving mice at the end of the holding period were destroyed. All tests were completed in duplicate.

The LD_{50} titration performed during each investigation on toxin void of halogens (control flask) involved the inoculation of 5 dilutions:

1:1600, 1:3200, 1:6400, 1:13,000 and 1:26,000 into lots of 6 mice each to determine the initial concentration of toxin present at the start of each evaluation.

Free available chlorine, combined chlorine, and chlorine dioxide determinations were made at intervals of 0, 2, 4, 8, 16, 30, and 60 minutes. No samples were taken at the 15-, 30-, and 60-second intervals because of the time element required to procure representative samples. Chloramine-T and Globaline determinations were made at the beginning and end of each investigation due to the large amount of sample required.

Hydrogen-ion concentration determinations were made at the beginning and end of each study.

The effect of halogens upon the rate of inactivation of the type A toxin was observed as 4 separate conditions: (1) effectiveness of available chlorine, (2) effectiveness of monochloramines, mixed monochloramine and dichloramine, and chloramine-T, (3) effectiveness of Globaline, and (4) effectiveness of chlorine dioxide. Types B, C, and D toxins were studied only in relation to their resistance to free available chlorine. Type E toxin was evaluated in terms of free available chlorine and chlorine dioxide efficacy.

RESULTS

TOXIN STABILITY

The stability of the partially purified types A and E toxins was determined in water buffered[12] at pH 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0 while stored at 25° C. The stability of the type E toxin was also studied at 5° C.

Samples of the type A toxin were taken daily throughout a 14-day period, followed by intermittent sampling thereafter, with the exception of the sample in water buffered at pH 11.0. At pH 11.0, samples were taken 3.0, 7.5, 15, 30, 60, and 120 minutes from the starting time of the examination. All samples were taken in quantities of 5 ml., placed in 1 ml of the designated neutralizer mixture, and inoculated in triplicate. Approximately 20,000 to 30,000 LD_{50}s per ml were present in each of the liter aliquots of water examined. The survival time of inoculated mice was used to assay the toxin concentration. The influence of pH upon the stability of the type A toxin can be seen in Table II. Except for the inconsistencies occurring in the case of pH 6.0, these data show a pattern of increasing instability of the toxin as pH is increased. The times required for destruction of the toxin at pH levels of 9.0, 10.0, and 11.0, were 28 days, 7 days, and 3 minutes, respectively. Although 100 percent destruction of the toxin at pH 11.0 has been reported in 3 minutes, the degree of destruction may have occurred sooner; however, samples were taken no earlier than 3 minutes after the investigation started.

Investigation of the stability of the type E toxin at both 5° and 25° C. at the above-mentioned pH levels was limited to 2 days due to the more rapid hydrolysis of the toxin. Table III indicates the relative stability of the toxin at 5° and 25° C. At 25° C., at least 50 percent destruction of the toxin at pH levels of 8.0, 9.0, 10.0, and 11.0 had taken place in less than one hour. Lowering the temperature to 5° C resulted in at least 15 percent destruction of toxin at pH levels of 9.0, 10.0, and 11.0 in 4 hours.

TABLE II. STABILITY OF PARTIALLY PURIFIED CL. BOTULINUM TYPE A TOXIN IN WATER AT VARIOUS pH LEVELS AND STORED AT 25°C.

Time	Average percentage toxin remaining after exposure					
	pH 6.0	pH 7.0	pH 8.0	pH 9.0	pH 10.0	pH 11.0
0 Min.	100.0	100.0	100.0	100.0	100.0	100.0
3 Min.	100.0	100.0	100.0	100.0	100.0	0.0
1 Day	100.0	100.0	100.0	67	13	--
2 Days	100.0	100.0	100.0	55	7	--
3 Days	100.0	100.0	100.0	39	0.8	--
4 Days	100.0	100.0	100.0	28	0.1	--
5 Days	60	100.0	100.0	28	0.05	--
6 Days	60	100.0	100.0	28	0.007	--
7 Days	60	100.0	100.0	28	0.0	--
10 Days	60	100.0	93	10	--	--
11 Days	60	100.0	46	5	--	--
14 Days	60	100.0	42	2	--	--
16 Days	60	100.0	42	0.2	--	--
18 Days	60	100.0	42	0.08	--	--
21 Days	37	100.0	42	0.05	--	--
23 Days	37	100.0	23	0.04	--	--
25 Days	37	100.0	10	0.01	--	--
27 Days	37	100.0	10	0.006	--	--
28 Days	37	100.0	2	0.0	--	--

TABLE III. STABILITY OF CL. BOTULINUM UNDIALYSED CRUDE TYPE E TOXIN IN WATER AT VARIOUS pH LEVELS AND TEMPERATURES.

Time	pH 6.0		pH 7.0		pH 8.0		pH 9.0		pH 10.0		pH 11.0	
	5°C	25°C	5°C	25°C	5°C	25°C	5°C	25°C	5°C	25°C	5°C	25°C
0 min.	100	100	100	100	100	100	100	100	100	100	100	100
½ min.	100	100	100	100	100	100	100	100	100	100	5	0.01
3 min.	100	100	100	100	100	100	100	L.A.	100	81	0.4	0.00
15 min.	100	100	100	100	100	60	100	46	100	*	0.00	0.00
30 min.	100	100	100	96	100	54	100	46	100	*		
1 hr.	100	100	100	74	100	35	100	21	40	0.2		
2 hr.	100	100	100	29	100	17	100	*	12	*		
4 hr.	100	100	100	10	100	3	85	0.4	7	0.03		
24 hr.	35	2.6	8	0.22	2.7	0.1	5.0	0.06	1	0.00		
48 hr.	34	*	4.7	0.20	1.5	0.1	0.15	0.03	0.1	0.00		

FREE AVAILABLE CHLORINE

Results of tests to determine the effectiveness of free available chlorine upon partially purified botulinum toxins at storage temperatures of 5° and 25° C., and pH levels of 7.0, 8.0, 9.0, and 10.0, are shown in Tables IV through VIII.

Tables IV and V show that when 0.5, 0.3, 0.22, and 0.1 mg/l free available chlorine were used, the rate of inactivation of demand-containing toxins was definitely related to the length of exposure and that the chlorine residual should be higher than the chlorine demand in order to produce toxin destruction. Therefore, 0.5 mg/l free available chlorine was used in all of the tests concerning the types A and D toxins. The chlorine demand of the types B and C toxins required initial addition of larger amounts of free chlorine. (Table VI). The type E toxins required initial addition of larger amounts of free available chlorine than the other four types of botulinum toxins. The chlorine demand and the apparent resistance to the action of free available chlorine of the type E toxins can readily be seen in Tables VII and VIII. The high chlorine demand of the type E toxins was due mainly to the presence of large amounts of ammonia-nitrogen (Table IX). When the type E toxins were dialysed, lower concentrations of free available chlorine were sufficient to obtain 99.9 to 99.99 percent inactivation.

COMBINED CHLORINE

Experimental evaluations of the effectiveness of combined chlorine (chloramines) upon the partially purified type A toxin at pH levels of 7.0 and 8.0, with storage temperatures of 5° and 25° C., can be seen in Table X.

TABLE IV. DESTRUCTION OF CL. BOTULINUM TYPES A AND D TOXINS BY FREE AVAILABLE CHLORINE AT pH 7.0 AND 25° C.

Character of toxin	Average percentage of toxin remaining after exposure to chlorine for						Average chlorine concentration (mg/l)			
							Initial		120 Sec.	
	15 Sec.	30 Sec.	60 Sec.	120 Sec.	240 Sec.	480 Sec.	Free	Total	Free	Total
Part. purified. Type "A"	0.80	0.00	.0.00	.0.0	0.0	0.0	0.50	0.50	0.21	0.21
" " " "	0.80	0.07	0.01	0.0	0.0	0.0	0.33	0.33	0.10	0.14
" " " "	2.00	0.30	0.06	0.0	0.0	0.0	0.22	0.22	0.00	0.00
" " " "	5.00	4.00	3.50	2.9	0.9	--	0.10	0.10	0.00	0.04
Pure Type "A"	0.80	0.05	0.02	0.0	0.0	0.0	0.15	0.15	0.11	0.12
Purified Type "D"	2.20	0.60	0.20	L.A.	0.02	0.0	0.17	0.17	0.11*	0.12*
" " "	0.15	<0.01	<0.01	0.0	0.0	0.0	0.54	0.54	0.44	0.45

L. A. Laboratory accident.

* Concentration present at 480 seconds.

TABLE V. DESTRUCTION OF CL. BOTULINUM TYPES A AND D TOXINS BY FREE AVAILABLE CHLORINE AT VARIOUS pH LEVELS AND TEMPERATURES.

Temp.	pH	Character of toxin	Average percentage of toxin remaining after exposure to chlorine for						Average chlorine concentration (mg/l)			
			15 Sec.	30 Sec.	60 Sec.	120 Sec.	240 Sec.	480 Sec.	Initial		120 Sec.	
									Free	Total	Free	Total
25° C	8.0	Part. purified Type "A"	0.03	0.00	0.00	0.00	0.0	0.0	0.52	0.52	0.21	0.21
"	9.0	" " "	0.01	0.00	0.00	0.00	0.0	0.0	0.57	0.57	0.29	0.34
"	10.0	" " "	<0.01	0.00	0.00	0.00	0.0	0.0	0.48	0.48	0.19	0.23
5° C	7.1	" " "	2.00	0.20	0.03	<0.01	0.0	0.0	0.49	0.49	0.24	0.36
"	8.0	" " "	5.00	1.00	0.02	<0.01	0.0	0.0	0.53	0.53	0.21	0.35
"	9.0	" " "	0.60	0.03	0.00	0.00	0.0	0.0	0.53	0.53	0.34	0.40
"	7.0	Purified Type "D"	2.00	0.70	0.12	<0.01	0.0	0.0	0.59	0.59	0.50	0.53

TABLE VI. DESTRUCTION OF (CL. BOTULINUM) PARTIALLY PURIFIED TYPES B AND C TOXINS BY FREE AVAILABLE CHLORINE AT pH 7.0 AND 25° C.

Type of Toxin	Average percentage of toxin remaining after exposure to chlorine for											Average chlorine concentration after exposure (mg/l)			
	0 Sec.	15 Sec.	30 Sec.	60 Sec.	120 Sec.	240 Sec.	480 Sec.	960 Sec.	1800 Sec.	3600 Sec.	4200 Sec.	Initial		120 Sec.	
												Free	Total	Free	Total
"B"	100	14	14	8	4	3.0	3.0	--	--	--	--	0.52	0.52	0.00	0.20
"B"	100	0.24	0.05	0.02	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.85	1.85	0.46	0.92
"C"	100	36	14	12	9	9.0	8.0	6.0	6.0	6.0	6.0	0.50	0.50	0.00	0.12
"C"	100	0.10	0.03	0.01	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.40	2.40	0.20	0.59
"C"	100	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.00	4.00	0.95	1.00

TABLE VII. DESTRUCTION OF UNDIALYSED, CRUDE, TYPE E TOXIN OF CL. BOTULINUM BY FREE AVAILABLE CHLORINE AT TWO pH LEVELS AND TEMPERATURES

pH	Temp. °C	Average percentage of toxin remaining after exposure to chlorine for										Average chlorine concentration (mg/l) after exposure			
												Initial		120 Sec.	
		0 Sec.	15 Sec.	30 Sec.	60 Sec.	120 Sec.	240 Sec.	480 Sec.	960 Sec.	1800 Sec.	3600 Sec.	Free	Total	Free	Total
7.0	25	100	100	100	100	100	100	100	48	48	24	0.55	0.55	0.00	0.12
"	"	100	100	100	57	40	40	40	19	--	2.4	4.30	4.30	0.09	2.50
"	"	100	86	86	63	63	63	25	16	16	2.0	7.30	7.30	0.26	5.40
"	"	100	16	16	16	13	4	1	0.2	0.03	—	17.0	20.0	0.24	20.0
"	"	100	0.5	0.3	0.15	0.08	0.0	0.0	0.0	0.00	--	25.0	32.0	0.10	31.0
"	"	100	0.3	0.17	0.06	0.00	0.0	0.0	0.00	0.00	--	40.0	40.0	0.87	37.0
"	5	100	3.0	3.0	0.6	0.6	0.6	0.4	0.06	0.03	—	29.0	34.0	0.04	33.0
10.0	25	100	0.6	0.5	0.14	0.07	0.05	0.02	0.00	0.00	--	27.0	31.0	0.14	29.0

TABLE VIII. DESTRUCTION OF Cl. BOTULINUM TYPE E TOXINS OF VARIOUS DEGREES OF PURITY BY FREE AVAILABLE CHLORINE AT pH 7.0 AND 25° AND 5°C.

Temp. °C.	Toxin Description	Average percentage of toxin remaining after exposure to chlorine for									Average Chlorine Concentration (mg/l) after exposure			
		0 Sec.	15 Sec.	30 Sec.	60 Sec.	120 Sec.	240 Sec.	480 Sec.	960 Sec.	1800 Sec.	Initial		120 Sec.	
											Free	Total	Free	Total
25	Undialysed calcium chloride treated toxin	100	100	100	100	100	59	11	8	5	2.90	2.90	0.00	2.40
"	Undialysed purified toxin (AP-II)	100	12	12	4	4	4	3	1	0.5	2.50	2.50	Trace	2.40
"	Undialysed purified toxin (AP-II)	100	10	6	6	6	2	1.1	0.3	0.1	5.30	5.30	0.08	5.30
"	Dialysed crude toxin	100	0.12	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.90	3.10	0.00	1.40
"	Dialysed purified toxin (AP-II)	100	1.1	0.2	0.05	0.0	0.0	0.0	0.0	0.0	0.50	0.50	0.22	0.35
"	" " " "	100	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.82	0.82	0.64	0.70
"	" " " "	100	0.6	0.2	0.02	0.0	0.0	0.0	0.0	0.0	0.92	0.92	0.23	0.53
"	" " " "	100	0.26	0.06	0.0	0.0	0.0	0.0	0.0	0.0	1.80	2.10	0.46	1.10
"	" " " "	100	<0.01	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.0	6.0	5.0	5.2
5	" " " "	100	12	4	0.3	0.1	0.05	0.03	0.01	0.0	0.52	0.52	0.28	0.35
"	" " " "	100	2.6	0.7	0.2	0.08	0.04	0.0	0.0	0.0	0.87	0.87	0.42	0.57

TABLE IX. INFLUENCE OF AMMONIA-NITROGEN UPON THE RATE OF INACTIVATION OF CL. BOTULINUM TYPE E. TOXINS, USING AN INITIAL CONCENTRATION OF 3 MG/L CHLORINE AT pH 7.0 AND 25°C.

Toxin Character*	NH_3-N (mg/l) in water containing toxin minus free available chlorine	NH_3-N (mg/l) in water containing toxin plus free available chlorine		Time required to produce 95% Inactivation of Toxin (in min.)
	0 seconds	20 sec.	15 min.	
Crude Undialysed	15.0	12.5	7.0	> 30
Calcium Chloride Treated Undialysed	8.5	8.5	4.0	> 30
AP-II Undialysed	0.2	0.15	0.10	8
Crude Undialysed	0.02	0.0	0.0	< 1
AP-II Dialysed	0.005	0.0	0.0	< 1

* Stage of Purification of Type E Toxin.

TABLE X. DESTRUCTION OF PARTIALLY PURIFIED TYPE A CL. BOTULINUM TOXIN BY COMBINED CHLORINE AT pHS' 7.0 AND 8.0 AND AT 25° AND 5°C.

Temp.	pH	Character of Combined Chlorine	Average percent toxin surviving after exposure								Average combined Cl_2 after exposure	
			0 min	3 min	7 min	15 min	30 min	1 hr.	2 hr.	4 hr.	0 mg/1 min.	4 hr.
25°C.	7.0	Mixed Mono- and Dichloramine	100	100	100	37	34	19	1.4	0.01	1.0	0.9
"	"	" " "	100	29	29	23	17	3	0.1	0.0	1.0	0.9
"	"	Monochloramine	100	100	100	100	100	100	100.0	65.0	0.9	0.7
"	"	" " "	100	62	53	37	37	27	8.0	8.0	2.1	1.9
"	"	" " "	100	48	48	33	12	12	6.0	0.7	4.1	3.4
"	"	" " "	100	25	11	7	7	3	2.0	0.2	5.9	5.5
"	"	" " "	100	25	25	24	14	10	3.0	0.04	11.6	10.0
"	8.0	" " "	100	100	100	100	70	50	26.0	23.0	11.2	10.0
5°C	7.0	" " "	100	43	43	39	35	23	23.0	1.3	11.6	11.3
"	8.0	" " "	100	100	100	100	80	73	73.0	27.0	11.6	10.3
25°C	7.0	Monochloramine-T	100	1	0.02	0.0	0.0	0.0	0.0	0.0	11.7	11.5

GLOBALINE

Results of the investigation to determine the effectiveness of a source of Globaline (iodine) upon partially purified type A toxin at pH levels of 6.0 to 8.7, and storage at 25° C., are shown in Tables XI and XII.

CHLORINE DIOXIDE

The effectiveness of chlorine dioxide was evaluated only on the various preparations of the type E toxin representing different stages of purification. The evaluations were carried out at pH 7.0, with storage at 5° and 25° C. Only one experiment concerned the inactivation of this toxin at pH 9.0. The results are shown in Table XIII. Chlorine dioxide does not react with ammonia as chlorine does and it was felt that chlorine dioxide might be more effective than chlorine in the presence of large amounts of ammonium sulfate. As shown in Table XIII, as compared with Tables VII and VIII, substantially lower doses of chlorine dioxide than chlorine were required to inactivate 99.9 to 99.99 percent of these preparations at pH 7.0.

DISCUSSION

EFFECT OF TEMPERATURE

Free Available Chlorine

The botulinum toxins were inactivated more slowly at 5° C than at 25° C when free available chlorine was present. Figure 2 illustrates the effects of the two test temperatures upon the types A (partially purified) and E dialysed purified) toxins containing little or no chlorine

TABLE XI. DESTRUCTION OF PARTIALLY PURIFIED TYPE A CL. BOTULINUM TOXIN, ACTION OF IODINE (GLOBALINE) AT pH 6.25 AND 8.10 AND STORED AT 25°C.

pH	Tap Water Hardness (mg/l Carbonate)	Average percent toxin persisting after exposure (seconds)								Ave. I_2 (mg/l) remaining after exposure (min.)	
		0	15	30	60	120	240	480	960	0	60
6.25	48	100	1.0	0.11	< 0.01	0.0	0.0	0.0	0.0	6.9	5.0
8.10	385	100	< 0.01	0.00	0.00	0.0	0.0	0.0	0.0	7.2	4.7
8.10	428	100	< 0.01	0.00	0.00	0.0	0.0	0.0	0.0	7.7	6.0

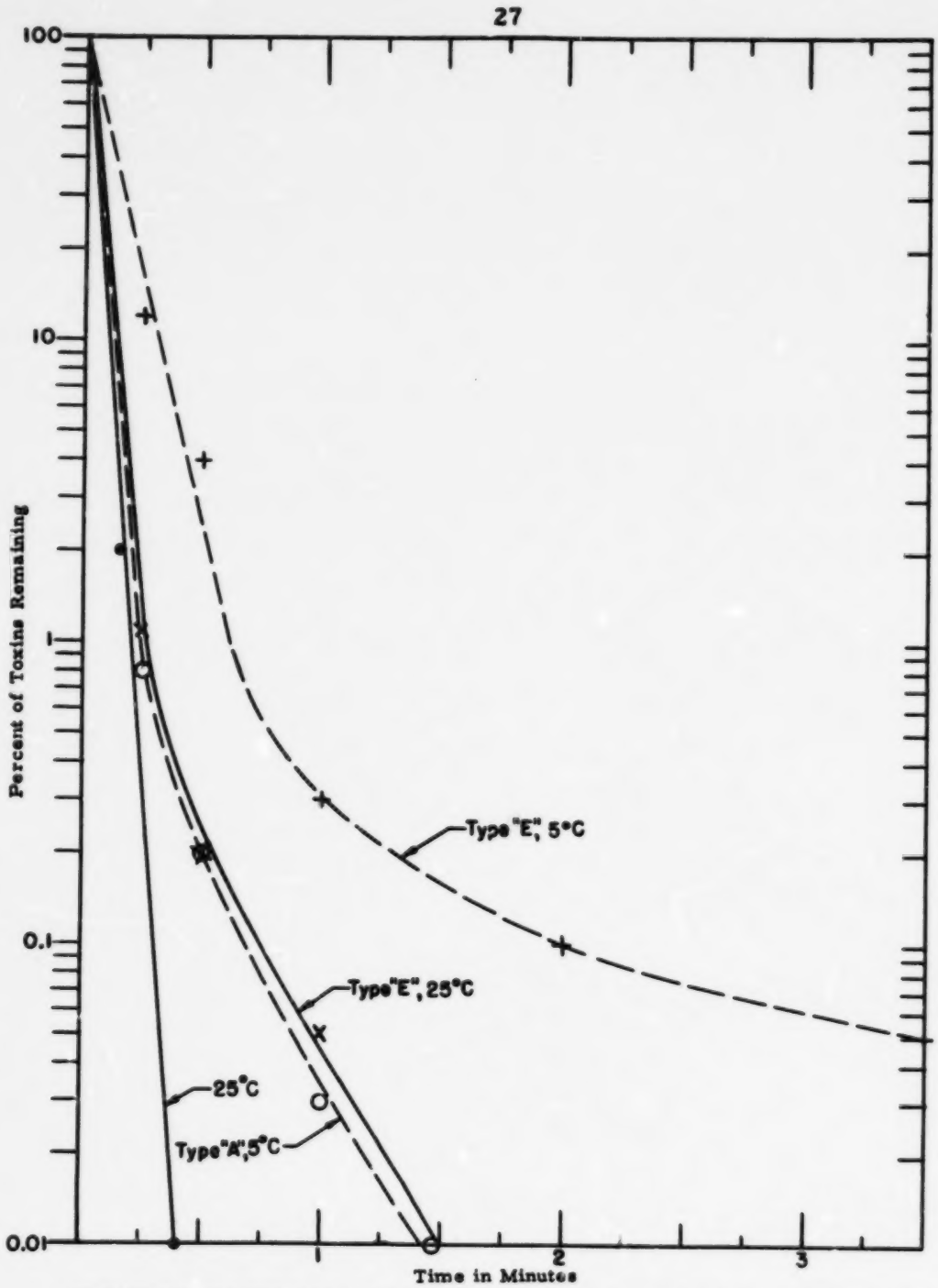
TABLE XII. EFFECT OF IODINE (GLOBALINE) UPON THE RATE OF INACTIVATION OF PARTIALLY PURIFIED TYPE A TOXIN AT 25° C., AND VARYING pH LEVELS.

Tap (Test) Water					Iodine (mg/l)		Initial LD50/ml test water	Calculated time for stated percent inactivation (seconds)		
pH(1)	pH(2)	Alkalinity HCO_3^- mg/l	Alkalinity $CO_3^=$ mg/l	Carbonate Hardness* mg/l	Initial	60 min.		50%	99.9%	99.99%
8.68	6.25	39	10	48	6.9	5.0	15,000	2	29	50
8.63	8.10	331	68	385	7.2	4.7	48,000	1	11	15
8.58	8.10	335	92	428	7.7	6.0	9,500	1	11	15

(1) Tap water alone.
(2) Tap water plus Globaline.
* Carbonate hardness caused by calcium bicarbonate and calcium bicarbonate alkalinity.

TABLE XIII. DESTRUCTION OF CL. BOTULINUM TYPE E TOXINS BY CHLORINE DIOXIDE AT pH 7.0 AND 9.0 AT 25°C AND 5°C.

Temp. °C.	Toxin Description	Average percentage of toxin remaining after exposure to chlorine for									Average total chlorine dioxide concentration (mg/l) after exposure		pH
		0 Sec.	15 Sec.	30 Sec.	60 Sec.	120 Sec.	240 Sec.	480 Sec.	960 Sec.	1800 Sec.	Initial	120 Sec.	
25	Undialysed crude toxin	100	0.3	0.3	0.3	0.3	0.3	0.3	0.09	0.09	0.90	0.12	7.0
"	" " "	100	0.055	0.044	0.035	0.024	0.012	0.0	0.0	0.0	1.9	0.18	"
5	" " "	100	0.07	0.06	0.06	0.05	0.0	0.0	0.0	0.0	1.8	0.17	"
25	Undialysed calcium chloride treated toxin	100	<0.02	0.00	0.00	0.00	0.0	0.0	0.0	0.0	1.2	0.09	"
"	" " "	100	<0.05	0.00	0.00	0.00	0.0	0.0	0.0	0.0	2.0	0.96	"
5	" " "	100	0.08	0.04	0.00	0.00	0.0	0.0	0.0	0.0	0.92	0.00	"
25	Undialysed purified toxin	100	<0.01	0.00	0.00	0.00	0.0	0.0	0.0	0.0	1.1	0.02	"
"	" " "	100	0.02	<0.01	0.00	0.00	0.0	0.0	0.0	0.0	1.90	1.6	"
5	" " "	100	<0.01	0.00	0.00	0.00	0.0	0.0	0.0	0.0	1.00	0.80	"
25	Dialysed crude toxin	100	0.4	0.4	0.24	0.00	0.0	0.0	0.0	0.0	0.49	0.11	"
"	" " "	100	<0.08	0.00	0.00	0.00	0.0	0.0	0.0	0.0	1.00	0.15	"
5	" " "	100	<2.0	0.00	0.00	0.00	0.0	0.0	0.0	0.0	0.45	0.08	"
25	Dialysed purified toxin	100	<0.01	0.00	0.00	0.00	0.0	0.0	0.0	0.0	0.41	0.27	"
5	" " "	100	0.05	<0.02	0.00	0.00	0.0	0.0	0.0	0.0	0.44	0.25	"
"	Undialysed purified toxin	100	0.02	<0.01	0.00	0.00	0.0	0.0	0.0	0.0	1.00	0.76	9.0



INFLUENCE OF TEMPERATURE UPON THE INACTIVATION OF CL. BOTULINUM TOXINS EXPOSED TO 0.5 MG/L APPLIED FREE AVAILABLE CHLORINE AT pH 7.0

FIGURE 2

demand at pH 7.0, when free available chlorine concentrations of approximately 0.5 mg/l were applied initially. In the majority of studies concerning the influence of temperature, the initial free available chlorine concentrations were approximately 0.5 mg/l.

At pH 7.0, 99.95 percent inactivation of the type A toxin occurred in 20 and 53 seconds at 25° and 5° C., respectively. Under the same conditions, the type E toxins required 60 and 210 seconds for 99.95 percent inactivation at 25° and 5° C., respectively.

The effect of temperature upon the inactivation of the type D purified toxin appeared to be greater than upon the partially purified type A toxin. At pH 7.0, 99.95 percent inactivation occurred at 22 and 81 seconds at 25° and 5° C., respectively.

Due to impurities in the types B and C toxins, the effects of temperature could not be evaluated when using an applied concentration of 0.5 mg/l chlorine.

Combined Chlorine

Lowering or raising the storage temperature resulted in pronounced differences in the amount of inactivation of the partially purified type A toxin. Table X shows the results obtained at pH 7.0, at both test temperatures, when the inactivation efficiency of monochloramine was evaluated. At 25° and 5° C., 1 and 2-2/3 hours respectively were required to inactivate 90 percent of the type A toxin.

Chlorine Dioxide

In the investigations involving chlorine dioxide, there appeared to be little or no difference in the degree of inactivation of the

dialysed purified type E toxin or in the time element for 99.9 to 99.99 percent inactivation when the test temperature was lowered 20°. Table XIII shows the influence of the different test temperatures upon the chlorine dioxide concentrations required to achieve inactivation of the various type E toxins.

Since Globaline was investigated only at 25° C. the effects of varying temperatures upon its inactivation of botulinum toxins are not available.

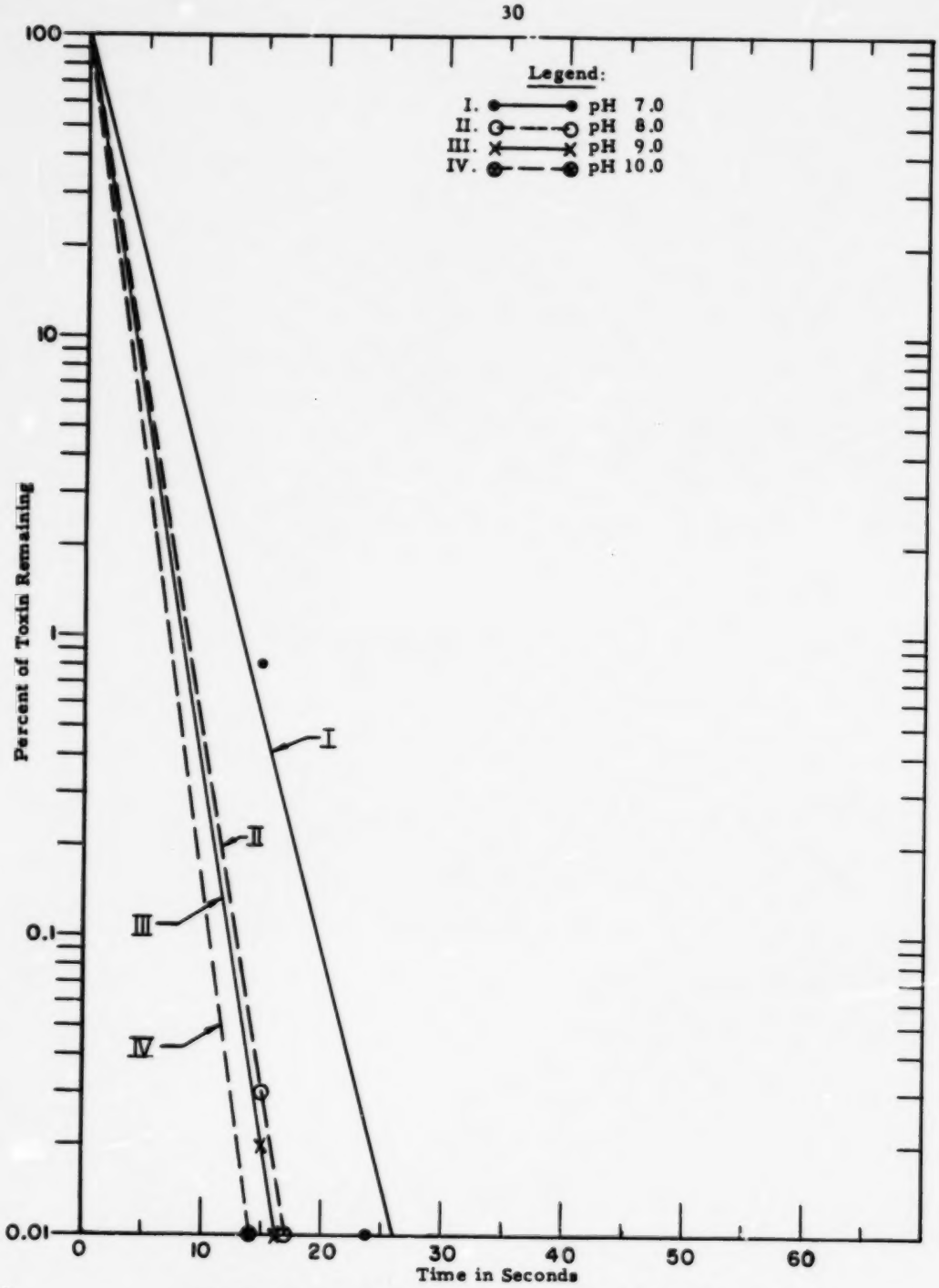
EFFECT OF pH

Free Available Chlorine

Stored test waters containing the partially purified type A toxin were evaluated in terms of percent inactivation by 0.5 mg/l applied free available chlorine at pH levels of 7.0, 8.0, 9.0, and 10.0. From the results obtained, it appeared that as the pH level increased from 7.0 to 10.0, the time required for 99.9 to 99.99 percent inactivation of the toxin decreased. The effect of varying hydrogen-ion concentrations upon inactivation can be seen in Figure 3. At pH 7.0, 8.0, 9.0, and 10.0, 0.5 mg/l of free available chlorine caused inactivation in 24, 17, 16, and 14 seconds, respectively, at 25° C.

Combined Chlorine

Inactivation of the partially purified type A toxin was decreased from 99.96 to 97.5 percent at the end of 4 hours, when the pH was raised from 7.0 to 8.0, in the presence of 11.6 mg/l monochloramine at 25° C. Increasing the pH of test waters containing toxins and monochloramine appeared to produce results of decreasing degrees of inactivation.



EFFECT OF pH UPON THE INACTIVATION OF PARTIALLY PURIFIED TYPE A TOXIN BY 0.5 MG/L APPLIED FREE AVAILABLE CHLORINE AT 25° C

FIGURE 3

Globaline

Increasing the pH level from 6.25 to 8.10 resulted in shortening the time required for 99.9 to 99.99 percent inactivation of the partially purified type A toxin in the presence of 6.85 to 7.7 mg/l iodine. Tables XI and XII show the effect of this change in pH upon the action of iodine at 25° C., using an exposure period of 8 minutes. At pH 8.10, 99.99 percent inactivation occurred in less than 15 seconds, while at pH 6.25, 40 seconds were required.

These results would appear to indicate that the hypoiodous acid was more efficient than the free iodine present, at the pH levels indicated, in inactivating the partially purified type A toxin.

Chlorine Dioxide

Information obtained at pH 7.0 and 9.0 at 5° C. appeared to indicate that the undialysed purified type E toxin was inactivated within the same period of time using an applied concentration of 1.1 mg/l chlorine dioxide. Due to the poor stability of the type E toxin at pH levels higher than 7.0, the inactivation was studied at 5° C.

EFFECT OF FREE AVAILABLE CHLORINE CONCENTRATION

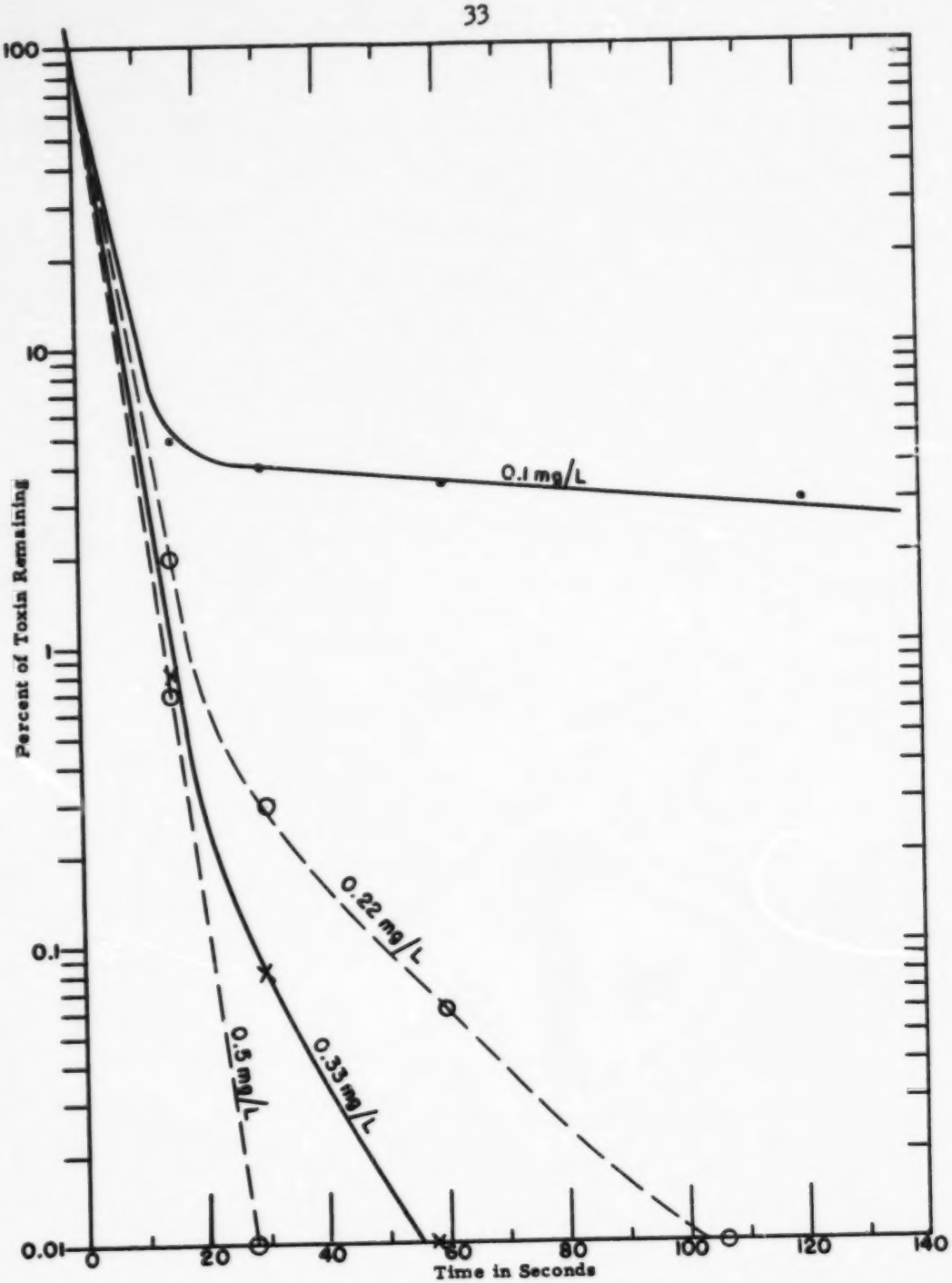
In the experiments on the inactivation of the partially purified type A toxin, periodic observations were made throughout a 30-minute holding period. It was interesting to note that as long as free available chlorine was present, inactivation proceeded rapidly. Where a sufficient chlorine demand existed, inactivation was impeded due to the conversion of free available chlorine to combined chlorine, or by the complete removal of total active chlorine. An initial free available chlorine

concentration of 0.1 mg/l produced 90 percent inactivation of the type A toxin in 6 seconds, but at the end of 240 seconds, 1 percent of the toxin still remained. Close examination revealed that at the end of 120 seconds, all of the free available chlorine had been converted to combined chlorine or had been completely removed from the test water. When an initial concentration of 0.5 mg/l free available chlorine was used, sufficient chlorine remained in the free available phase, at the end of 120 seconds at 25° C., to inactivate at least 99.99 percent of the toxin. Tables IV to IX show the effects of demand-containing toxins upon the initial applied free available chlorine concentrations.

Figure 4 illustrates the inactivation of the partially purified type A toxin in 28, 56, and 103 seconds, respectively, by initial applied free available chlorine concentrations of 0.5, 0.33, and 0.22 mg/l.

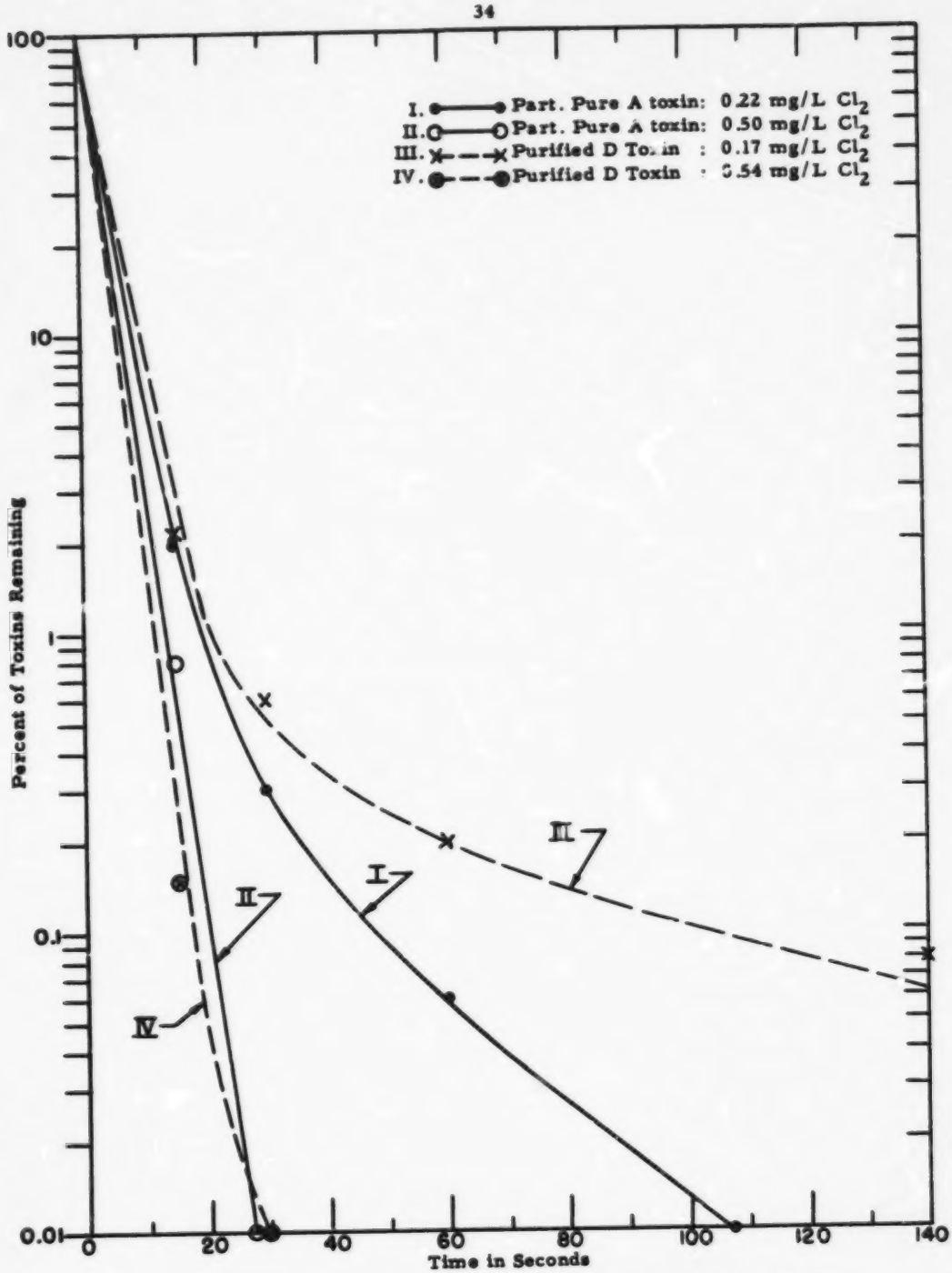
The influence of varying free available chlorine concentrations upon 99.9 to 99.99 percent inactivation of the partially purified type A and purified type D toxins at pH 7.0 and 25° C. within 120 seconds can be seen in Figure 5 and also in Table IV. Inactivation of the partially purified type A toxin and the purified type D toxin, using initial applied concentrations of 0.22 and 0.17 mg/l free available chlorine, required 106 and 294 seconds, respectively. When 0.5 and 0.54 mg/l applied free available chlorine were used initially, the types A and D toxins required 27 and 30 seconds, respectively, for 99.99 percent inactivation.

Thus the data indicated that the type D toxin was more resistant to the action of free available chlorine than the type A toxin. In



EFFECT OF VARYING CONCENTRATIONS OF APPLIED FREE AVAILABLE CHLORINE UPON THE SURVIVAL OF PARTIALLY PURIFIED TYPE A TOXIN AT pH 7.0 AND 25° C

FIGURE 4



EFFECT OF VARYING CONCENTRATIONS OF APPLIED FREE AVAILABLE CHLORINE UPON THE SURVIVAL OF TYPES A AND D TOXINS AT pH 7.0 AND 25° C

FIGURE 5

contrast to the chlorine demand of the partially purified type A toxin, the type D toxin persisted 294 seconds before 99.99 percent inactivation occurred, using an initial applied free available chlorine concentration of 0.17 mg/l, and there still remained 0.11 mg/l free available chlorine at the end of 480 seconds (Table IV).

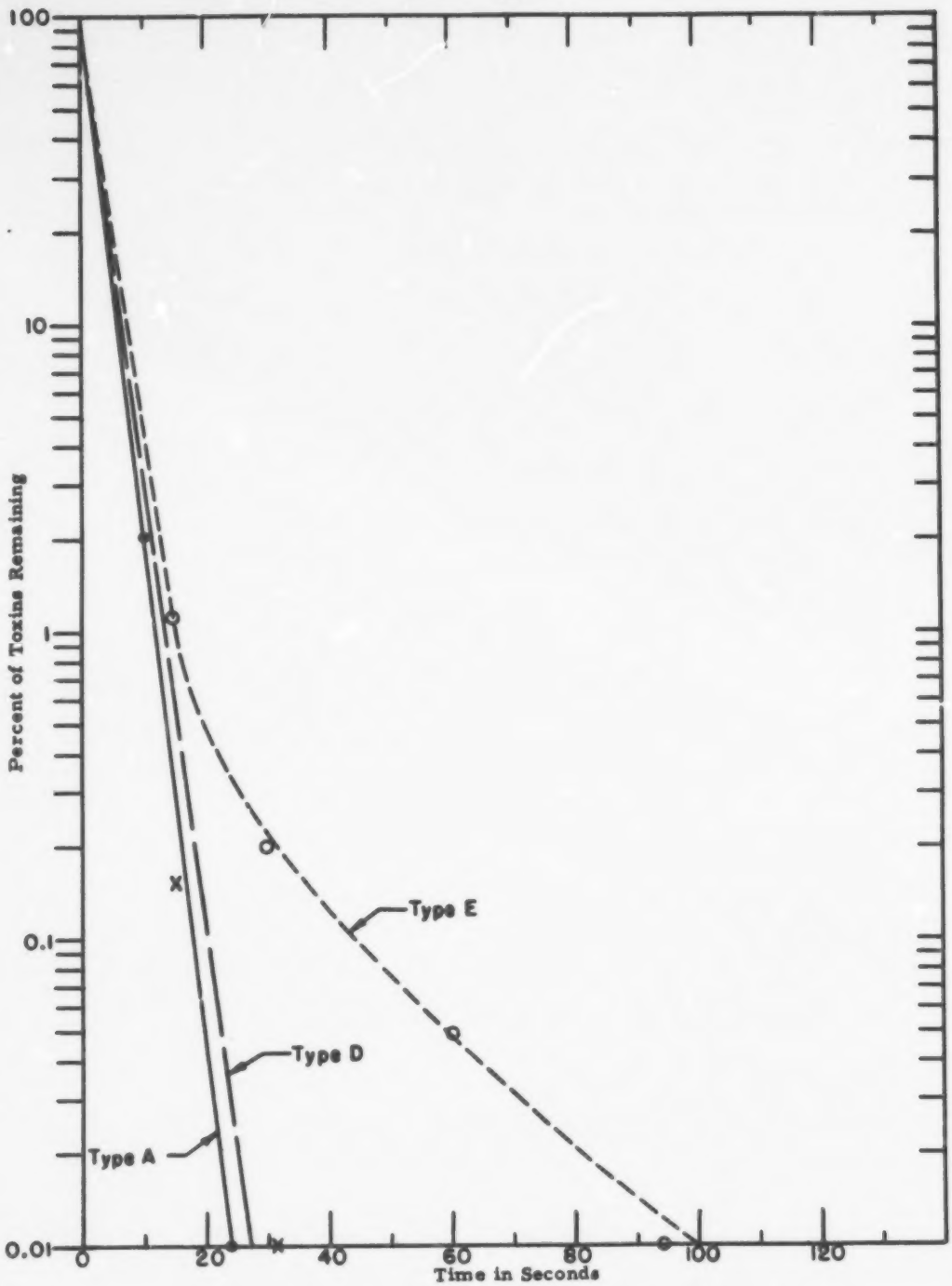
Several different preparations of the type E toxin exhibited greater degrees of resistance to free available chlorine than all of the other botulinum toxins investigated, as can be seen in Tables VII and VIII. The purified type E toxin was not inactivated rapidly when a free available chlorine residual of 0.22 mg/l remained at the end of 120 seconds, especially when compared to the inactivation of the other types of toxins.

Figure 6 illustrates the resistance of the purified types A, D, and E toxins to the action of 0.5 mg/l applied free available chlorine at pH 7.0 and 25° C. Note that the types of A,D, and E toxins required 18, 21, and 44 seconds for 99.9 percent inactivation, respectively. Types B and C have been omitted from this graph since they show relatively low resistance to free available chlorine.

EFFECT OF COMBINED CHLORINE

The influence of combined chlorine was evaluated under 3 conditions: (1) a mixture containing 95 percent monochloramine and 5 percent dichloramine, prepared by using a 250:1 molar ratio of ammonia to chlorine; (2) 100 percent monochloramine, prepared by using a 1,000:1 molar ratio of ammonia to chlorine; and (3) monochloramine-T, prepared from a commercial source.

The results shown in Table X appear to indicate that the mixture of monochloramine and dichloramine was the most effective inorganic



EFFECT OF 0.5 MG/L APPLIED FREE AVAILABLE CHLORINE UPON THREE CL. BOTULINIUM TOXINS AT pH 7.0 AND 25° C

FIGURE 6

chloramine compound for inactivating the partially purified type A toxin. Since maintenance of the proper pH reaction affects the percentage of mono- and dichloramine present under specified conditions of preparation, the ratio between the two forms could not be reliably maintained. Utilization of the 100 percent monochloramine as a more reliable compounded mixture was made in the remainder of the experiments. Concentrations of 1.0, 5.9, and 11.6 mg/l applied monochloramine produced 25, 99.8, and 99.96 percent inactivation, respectively, of the partially purified type A toxin in 4 hours when held at pH 7.0 and 25° C. When held at pH 8.0 under the same conditions, the type A toxin was inactivated by 97.5 percent in 4 hours, using 11.6 mg/l monochloramine.

In those studies utilizing monochloramine-T, 99.98 percent inactivation of the partially purified type A toxin occurred in 7 minutes at pH 7.0 and 25° C. These results appear to indicate that the organic monochloramine-T was far more effective than inorganic monochloramine in producing inactivation of the type A toxin using equal concentrations of 11.6 mg/l.

EFFECT OF IODINE CONCENTRATION

All of these experiments were made using one level of iodine (Globaline) concentration. Although the concentration varied slightly between 6.9 and 7.7 mg/l iodine, this difference was not thought to be significant in terms of the results of inactivation observed. Preparation of the iodine concentration in the test water was accomplished by dissolving one Globaline tablet per quart of sterilized tap water. The

stability of the tablets affected the final iodine concentration in the test waters used.

The data in Tables XI and XII appear to indicate that in waters of low carbonate hardness (48 mg/l), 6.9 mg/l iodine produced greater than 99.99 percent inactivation of the type A partially purified toxin in 60 seconds at pH 6.25 and 25° C. In waters of high carbonate hardness (385 to 430 mg/l), 7.2 to 7.7 mg/l iodine produced greater than 99.99 percent inactivation in less than 15 seconds at pH 8.1 and 25° C. No explanation is offered why a shorter length of time for inactivation was required for test water containing a greater degree of carbonate hardness.

EFFECT OF CHLORINE DIOXIDE CONCENTRATION

The relation of chlorine-combining substances to apparent increased resistance of toxins suggested the use of chlorine dioxide, which is relatively unaffected by the presence of ammonium salts. During the evaluation of the type E toxin inactivation by free available chlorine, the presence of ammonia sulfate in the undialysed preparations necessitated very high initial concentrations of free available chlorine to inactivate this toxin. When dialysed type E toxin preparations were used, impurities adversely affected the efficiency of free available chlorine, but to a lesser degree.

Table XIII shows the effects of chlorine dioxide upon the type E toxin preparations at pH 7.0 and 25° C. As shown in this table, initial chlorine dioxide concentrations of 0.45 to 2.0 mg/l produced 99.9 percent inactivation of all toxin preparations within 10 to 16 seconds. By

reference to Tables VII, VIII, and XIII, an initial applied concentration of 40 mg/l free available chlorine was slightly less effective than an initial applied concentration of 1.9 mg/l chlorine dioxide in inactivating the undialysed, crude type E toxin at pH 7.0 and 25 ° C., in approximately the same period of time. At the end of 120 seconds exposure to the crude toxin, only 0.87 mg/l free available chlorine remained as compared to 0.18 mg/l chlorine dioxide.

In all of the previous investigations involving the efficiency of chlorine dioxide and free available chlorine, measurement of the concentrations present appeared to be the greatest obstacle. By utilization of a new method[18] for determining chlorine dioxide under the conditions of this experiment, the readings obtained were considered to be more reliable. Some questions remain regarding the general use of this method in natural waters containing a variety of interfering substances. On the other hand, measurement of the free available chlorine concentration, in the presence of strong chlorine-combining and/or demanding substances, by accepted standard procedures[12] proved unsatisfactory. As a result, modified procedures similar to Moore's[19] methods of using correction factors in the O.T.A. test were utilized which appeared to produce readings more indicative as regards inactivation of the type E toxin by free available chlorine.

VARIATIONS IN TOXIN SENSITIVITY

The pure and partially purified types A, B, C, D, and E toxins of Cl. botulinum were all evaluated in terms of resistance to free available chlorine at pH 7.0 and 25° C. The resistance of the five toxins investigated

to free available chlorine at 25° C and pH 7.0 appears to occur in this order: E> B> C> D> A. There are insufficient data present, due mainly to the poor stability of the toxins, to indicate the exact relationship that exists for all five toxins at pH levels 8.0, 9.0, and 10.0 with storage at 25° and 5° C.

CONCLUSIONS

The inactivation of Cl. botulinum toxins by free available chlorine has been shown to occur as long as a residual of free available chlorine remains in the water being evaluated. Hydrogen-ion concentration between pH 7.0 and 10.0 does not effect markedly the rate of destruction of the toxins by free available chlorine. Lowering the temperature from 25° to 5° C. approximately triples the time required for 99.9 to 99.9 percent destruction of the toxins. Free available chlorine concentrations less than 0.5 mg/l were not able to satisfy the chlorine demand of the partially purified types B, C, and E toxins. At pH levels of 7.0 through 10.0, 0.5 mg/l free available chlorine caused 99.9 to 99.99 percent inactivation of the partially purified type A toxin in 15 to 30 seconds at 25° C.

Chloramines are relatively ineffective in destroying botulinum toxins. Inactivation of the partially purified type A toxin by solutions containing 95 percent monochloramine, 5 percent dichloramine, and 100 percent monochloramine, was affected by changes in the hydrogen-ion concentration, temperatures, and length of exposure time.

The action of iodine was similar to free available chlorine in terms of time required to produce a 99.9 to 99.99 percent inactivation

of the partially purified type A toxin. In the determinations of the effectiveness of iodine, Globaline tablets, imparting 8 mg/l iodine when dissolved in one quart of water, were used. It is most probable that in terms of equal concentrations (mg/l) of iodine and free available chlorine, the latter would be more effective in producing rapid inactivation of the toxins.

Chlorine dioxide appears to be somewhat superior to free available chlorine in destroying botulinum toxin. Uncertainty as to the bactericidal effectiveness of chlorine dioxide together with questions on the analytical method for chlorine dioxide in natural waters make it unwise to recommend its use as a general protective measure against biological agents in place of chlorine.

From the data presented, it appears that 99.9 to 99.99 percent inactivation of the Cl. botulinum toxins, types A, B, C, D, and E will occur when a sufficient residual of titrable free available chlorine (not less than 1.0 mg/l) exists in the water supply being evaluated for at least 5 minutes.

REFERENCES

1. Lamanna, C., McElroy, O. E., and Eklund, H. W. 1946 The purification and crystallization of Clostridium botulinum type A toxin. Science, 102:613-614.

2. Lamanna, C., Eklund, H. W., and McElroy, O. E. 1946 Botulinum toxin (type A), including study of shaking with chloroform as step in isolation procedure. Jour. Bact., 52:1-13.

3. Abrams, A., Kegeles, G., and Hottle, G.A. 1946 The purification of toxin from Clostridium botulinum, type A. Jour. Biol. Chem., 164:63-79.

4. Duff, J. T., Wright, G. G., Klerer, J., and Moore, D. E. 1952 A simplified procedure for purification of Clostridium botulinum type A toxin. Bact. Proc., 107.

5. Boor, A. K., Tresselt, H. B., and Schantz, E. J. 1955 Effects of salts and colloids on potency of botulinum toxin. Proc. Soc. Exp. Biol. and Med., 89:270-72.

6. Lamanna, C., Jensen, W. I., and Bross, I.D.S. 1955 Body weight as a factor in the response of mice to botulinal toxins. Amer. Jour. Hyg., 62:21-28.

7. Lowenthal, J. P. and Lamanna, C. 1951 Factors affecting botulinal hemagglutination and relationship between hemagglutinating activity and toxicity of toxin preparations. Amer. Jour. Hyg., 54:342-353.

8. Sherman, J. M., Stark, C. N., and Stark, P. 1928 Destruction of botulinus toxin by milk bacteria. Jour. Dairy Sci., 11:352-358

9. Brygoo, E. R. 1953 Resistance of botulinus toxins to dilution in water. Ann. Inst. Pasteur, 84:1039-1040.

10. Brygoo, E. R. 1953 Action of disinfectants on botulinus toxins diluted in water. Ann. Inst. Pasteur, 84:1040-41.

11. D'Arca, S. and D'Arca, S. A. 1955 Further studies on the detoxifying power of free active chlorine with respect to type A botulinum toxin (Ancora sul potere svelenate del chloro attivo libero sulla tossina botulinica tipo A.) Nuovi Annali D'Igiene e Microbiologia, 4:355-360.

12. Standard Methods for the Examination of Water and Sewage. 1946 Amer. Public Health Assoc. andAmer. Water Works Assoc., New York, 9th edit.

13. Brazis, A. R., Leslie, J. E., Kabler, P. W., and Woodward, R. L. 1957 Sporicidal action of free available chlorine. Special Report to Department of the Navy, Bureau of Yards and Docks, Project NY 300 010 Sub-project 6, April.

14. Ridenour, G. M. and Ingols, R. S. 1947 Bactericidal properties of chlorine dioxide. Jour. A.W.W.A., 39:561-567.

15. Ridenour, G. M. and Armbruster, E. H. 1949 Bactericidal effect of chlorine dioxide. Jour. A.W.W.A., 41:537-550.

16. Ridenour, G. M. and Ingols, R. S. 1946 Inactivation of poliomyelitis virus by "free chlorine". Amer. Jour. Public Health, 36:639-644.

17. Ridenour, G. M., Ingols, R. S., and Armbruster, E. H. 1949 Sporicidal properties of chlorine dioxide. Water and Sewage Works, 96:1-5.

18. Post, M. and Moore, W. Allan. 1958 Determination of chlorine dioxide in treated surface waters. Analytical Chemistry, In Press.

19. Moore, E. W. 1951 Fundamental of chlorination of sewage and waste. Water and Sewage Works, 98:130-136.